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Margaret M Mesa

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :

Ivarie et al. :

Application No: 09/173,864 : Group Art Unit: 1633

Filed: October 16, 1998 : Examiner: Sumesh Kaushal

Title: NOVEL TRANSGENIC BIRDS AND THEIR EGGS

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION UNDER 37 CFR 1.132

I, Jeffrey Rapp, Ph.D., hereby declare as follows:

1. I am a staff scientist employed by AviGenics, inc., of Athens, GA., licensee of the subject application, since June 1999. I consider myself to be skilled in the art of avian transgenesis. My curriculum vitae is appended hereto.

2. The following reports experiments conducted by me personally, or by those working under my supervision, to produce a transgenic chicken expressing an exogenous protein, following the procedures set forth in the subject application of Ivarie, *et al.*

3. One set of experiments was designed to demonstrate the expression of human interferon in a chicken, as suggested at page 32, line 6 of the specification.

4. Following the teachings of Example 1 (Vector Construction) of the specification, an pNLB-CMV-IFN vector was created, substituting an IFN encoding sequence for the BL encoding sequence of the Example.

The DNA sequence for human interferon α-2b based on hen oviduct optimized codon usage was created using the BACKTRANSLATE program of the Wisconsin Package, version 9.1 (Genetics Computer Group. Inc., Madison, WI) with a codon usage table compiled from the chicken (*Gallus gallus*) ovalbumin, lysozyme, ovomucoid, and ovotransferrin proteins. The template and primer oligonucleotides listed in Table 1 were amplified by PCR with *Pfu* polymerase (Stratagene, La Jolla, CA) using 20 cycles of 94°C for 1 min., 50°C for 30 sec., and 72°C for 1 min. and 10 sec. PCR products were purified from a 12% polyacrylamide-TBE gel by the "crush and soak" method (Maniatis *et al.* 1982), then combined as templates in an amplification reaction using only IFN-1 and IFN-8 as primers. The resulting PCR product was digested with *Hind* III and *Xba* I and gel purified from a 2% agarose-TAE gel, then ligated into *Hind* III and *Xba* I digested, alkaline phosphatase-treated pBluescript KS (Statagene), resulting in the plastmid pBluKSP-IFNMagMax. Both strands were sequenced by cycle sequencing on an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, CA) using

universal T7 or T3 primers. Mutations in pBluKSP-IFN derived from the original oligonucleotide templates were corrected by site-directed mutagenesis with the Transformer Site-Directed Mutagenesis Kit (Clontech, Palo Alto, CA). The IFN coding sequence was then removed from the corrected pBluKSP-IFN with *Hind* III and *Xba* 1, purified from a 0.8% agarose-TAE Gel, and ligated to *Hind* III and *Xba* I digested, alkaline phosphatase-treated pCMV-BetaLa-3B-dH. The resulting plasmid was pCMV-IFN which contained IFN coding sequence controlled by the cytomegalovirus immediate early promoter/enhancer and SV40 polyA site. To clone the IFN coding sequence controlled by the CMV promoter/enhancer into the NLB retroviral plasmid, pCMV-IFN was first digested with *Cla*I and *Xba*I, then both ends were filled in with Klenow fragment of DNA polymerase (New England BioLabs, Beverly, MA). pNLB-adapter was digested with *Nde*I and *Kpn*I, and both ends were made blunt by T4 DNA polymerase (New England BioLabs). Appropriate DNA fragments were purified on a 0.8% agarose-TAE gel, then ligated and transformed into DH5α cells. The resulting plasmid was pNLB-adapter-CMV-IFN. This plasmid was then digested with *Mlu*I and partially digested with *Blp*I and the appropriate fragment was gel purified. pNLB-CMV-EGFP was digested with *Mlu*I and *Blp*I, then alkaline-phosphatase treated and gel purified. The *Mlu*I/*Blp*I partial fragment of pNLB-adapter-CMV-IFN was ligated to the large fragment derived from the *Mlu*I/*Blp*I digest of pNLB-CMV-EGFP, creating pNLB-CMV-IFN.

5. Following the procedures of Example 2 (Production of Transduction Particles), transduction particles of pNLB-CMV-IFN were produced.

Senta packaging cells (Cosset *et al.*, 1990) were plated at a density of 3 x 10^5 cells/35mm tissue culture dish in F-10 medium (Life Technologies) supplemented with 50%

calf serum (Atlanta Biologicals), 1% chicken serum (Life Technologies), 50 μg/ml hygromycin (Sigma), and 50 μg/ml phleomycin (CAYLA, Toulouse, France). These cells were transfected 24h after plating with 2 μg of CsCl-purified pNLB-CMV-IFN DNA and 6 μl of Lipofectin liposomes (Life Technologies) in a final volume of 500 μl Optimem (Life Technologies). The plates were gently rocked for four hours at 37° C in a 5% CO_2 incubator. For each well, the media was removed, washed once with 1 ml of Optimem and re-fed with 2 mls of F-10 medium supplemented with 50% calf serum, 1% chicken serum, 50 μg/ml hygromycin, and 50 μg/ml phleomycin. The next day, medium from transfected Sentas was recovered and filtered through a 0.45 micron filter. This medium was then used to transduce Isolde cells. 0.3 ml of the filtered medium recovered from Senta cells was added to 9.6 ml of F-10 (Life Technologies) supplemented as described above, in addition to polybrene (Sigma) at a final concentration of 4 μg/ml. This mixture was added to 10^6 Isolde packaging cells (Cosset *et al.*, 1990) plated on a 100mm dish the previous day, then replaced with fresh F-10 medium (as described for Senta growth) 4h later. The next day, the medium was replaced with fresh medium which also contained 200 μg/ml neomycin (G418, Sigma). Every other day, the medium was replaced with fresh F- 10 medium supplemented with 50% calf serum, 1% chicken serum, 50 μg/ml hygromycin, 50 μg/ml phleomycin, and 200 μg/ml neomycin. Eleven to twelve days later, single colonies were visible by eye, and these were picked and placed into 24 well dishes. When some of the 24 well dishes became confluent, medium was harvested and titered to determine the cell lines with the highest production of retrovirus. Titering was performed by plating 7.5 x 10^4 Senta cells per well in 24 well plates on the day prior to viral harvest and transduction. The next day 1.0 ml of fresh F-10 medium

supplemented with 50% calf serum, 1% chicken serum, 50 µg/ml hygromycin, and 50 µg/ml phleomycin was added to each well of the isolated Isolde colonies. Virus was harvested for 8-10 hours. The relative density of each well of Isoldes was noted. After 8-10 hours, 2 and 20µl of media from each well of Isoldes was added directly to the media of duplicate wills of the Sentas. Harvested medium was also tested for the presence of IFN by IFN ELISA and for IFN bioreactivity. The next day the media was replaced with F- 10 medium supplemented with 50% calf serum, 1% chicken serum, 50 µg/ml hygromycin, 50 µg/ml phleomycin, and 200 µg/ml neomycin. When obvious neomycin resistant colonies were evident in the wells of transduced Sentas, the number of colonies was counted for each well. The Isolde colony producing the highest titer was determined by taking into account the number of colonies and correcting for the density of the Isolde cells when the viral particles were harvested (i.e., if two Isolde colonies gave rise to media with the same titer, but one was at a 5% density abd the other was at a 50% density at the time of viral harvest, the one at the 5% density was chosen for further work, as was the case in the present example).

The Isolde cell line producing the highest titer of IFN-encoding transducing particles was scaled up to six T-75 tissue culture flasks. When flasks were confluent, cells were washed with F-10 medium (unsupplemented) and transducing particles were then harvested for 16h in 14 ml/flask of F-10 containing 1% calf serum (Atlanta Biologicals) and 0.2% chicken serum (Life Technolocyies). Medium was harvested, filtered through a 0.45 micron syringe filter, then centrifuged at 195,000xg in a Beckman 60Ti rotor for 35 min. Liquid was removed except for 1 ml, and this was incubated with the pellet at 37°C with gentle shaking for one hour. Aliquots were frozen at -70°C. Transducing particles were then titered on Senta cells to determine concentrations used to inject embryos.

6. Following the procedures of Example 3 (Production of Transgenic Chickens), chimeric birds were produced.

Approximately 300 White Leghorn (strain Line 0) eggs were windowed according to the Speksnijder procedure (U.S. Patent No. 5,897,998), then injected with ~7 x 10^4 transducing particles per egg. Eggs hatched 21 days after injection, and human IFN levels were measured by IFN ELISA from serum samples collected from chicks one week after hatch.

7. Following the procedures of Example 10 (Production of Fully Transgenic G1 Chickens), males were selected for breeding.

To screen for G_0 roosters which contained the IFN transgene in their sperm, DNA was extracted from rooster sperm samples by Chelex-100 extraction (Walsh *et al.*, 1991). DNA samples were then subjected to Taqman™ analysis on a 7700 Sequence Detector (Perkin Elmer) using the "neo for-1" (5'-TGGATTGCACGCAGGTTCT-3') and "neo rev-1" (5'-GTGCCCAGTCATAGCCGAAT-3') primers and FAM labeled NEO-PROBE1 (5'-CCTCTCCACCCAAGCGGCCG-3') to detect the transgene. Three G_0 roosters with the highest levels of the transgene in their sperm samples were bred to nontransgenic SPAFAS (White Leghorn) hens by artificial insemination.

Blood DNA samples were screened for the presence of the transgene by Taqman™ analysis as described above. Out of 1,597 offspring, one rooster was found to be transgenic (a.k.a. "Alphie"). Alphie's serum was tested for the presence of hIFN by hIFN ELISA, and hIFN was present at 200 nanograms/ml.

Alphie's sperm was used for artificial insemination of nontransgenic SPAFAS (White Leghorn) hens. To date. 106 out of 202 (~52%) offspring contain the transgene as detected by Taqman™ analysis. These breeding results follow a Mendelian inheritance pattern and indicate that Alphie is transgenic.

Alphie was mated with nontransgenic White Leghorn hens to produce G_2 offspring, and blood DNA from G_2 chicks was assayed by Taqman analysis as described above. Of the 245 chicks tested, 149 (60.8%) contained the transgene, indicating Mendelian inheritance of the transgene. Egg white material from 150 of the G_2 hens has been assayed to date for hIFN by ELISA and found to contain an average of about 900 nanograms/ml hIFN. G_2 hens that did not contain the transgene, as determined by Taqman analysis, also did not contain hIFN in their egg white.

8. In other experiments, additional proteins were produced in transgenic chickens, including GM-CSF, human erythropoietin (EPO), and β-lactamase (β-lac), as suggested on page 32, lines 3-13, of the subject application of Ivarie, *et al.* These additional proteins were produced using the procedures as set forth above (pNLB-CMV-GMCSF and pNLB-CMV- β-lac vectors), except that the promoter used for the production of EPO was a synthetic promoter, MDOT, constructed as suggested on page 11, lines 12-19, and having both ovomucoid and conalbumin sequence elements (pNLB-MDOT-EPO vector).

9. G_0 birds containing the EPO transgene were tested to determine expression levels of EPO in serum. Of the 62 birds tested (60 experimental birds and 2 controls), 23 were positive for EPO expression in the serum with levels $\geq$ 3.0 ng/ml. The additional 27 birds had EPO levels

ranging from 0.058 ng/ml to 2.450 ng/ml. The two control birds were negative for EPO expression.

In a similar manner, serum samples were assayed by ELISA for GM-CSF production in 73 G_0 birds, produced as described above. Results showed that 3 of the experimental birds had serum levels of GM-CSF $\geq$ 1.0 ng/ml, while an additional 11 birds had levels ranging from 0.026 ng/ml to about 1.82 ng/ml. The remainder of the birds, and the three controls, were negative for GM-CSF production.

10. Consistent expression of β-lactamase was shown in the serum of G_0, G_1, and G_2 transgenic chickens and in the egg white of eggs from G_2 hens, produced according to the above-described procedures. Representative results, for example, showed serum expression in G_1 Hen 5657, and her G_2 progeny, of about 2.0 μg/ml of β-lactamase. Both G_1 rooster 4133 and his G_2 progeny demonstrated levels of β-lactamase in serum samples of about 6.0 μg/ml. β-lactamase in egg whites, from eggs of G_2 hens sired by rooster 4133, was expressed in levels ranging from about 0.6 μg/ml to about 1.2 ug/ml.

This declaration is made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon. All statements made of my own knowledge are true and all statements made on information and belief are believed to be true.

Jeffrey Rapp

Jeffrey Rapp, Ph.D.

Table 1. Oligonucleotides used for IFN gene synthesis

Template	Sequence of Template	Primer 1	Sequence of Primer 1	Primer 2	Sequence of Primer 2
IFN-A	5'-ATGGCTTTGACCTTTGCCTTACTGGTGGCTCTCCTGGTGCTGAGCTGCAAGAGCAGCTGCTCTGTGGGCTGCGATCTGCCTCA-3'	IFN-1	5'-CCCAAGCTTTCACCATGGCTTTGACCTTTGCCTT-3'	IFN-2	5'-CTGTGGGTCTGAGGCAGAT-3'
IFN-B	5'-GACCCACAGCCTGGGCAGCAGGAGGACCCTGATGCTGCTGGCTCAGATGAGGAGAATCAGCCTGTTTAGCTGCCTGAAGGATAGGCACGATTTTGGCTTT-3'	IFN-2b	5'-ATCTGCCTCAGACCCACAG-3'	IFN-3b	5'-AACTCCTCTTGAGGAAAGCCAAAATC-3'
IFN-C	5'-CTCAAGAGGAGTTTGGCAACCAGTTTCAGAAGGCTGAGACCATCCCTGTGCTGCACGAGATG-3'	IFN-3c	5'-GATTTTGGCTTTCCTCAAGAGGAGTT-3'	IFN-4	5'-ATCTGCTGGATCATCTCGTGC-3'
IFN-D	5'-TCCAGCAGATCTTTAACCTGTTTAGCACCAAGGATAGCAGCGCTGCTTGGGATGAGACCCTGCTGGATAAGTTTTACACCGAGCTGTACCAGCA-3'	IFN-4b	5'-GCACGAGATGATCCAGCAGAT-3'	IFN-5	5'-ATCGTTCAGCTGCTGGTACA-3'
IFN-E	5'-CTGAACGATCTGGAGGCTTGCGTGATCCAGGGCGTGGGCGTGACCGAGACCCCTCTGATGAAGGAGGATAGCATCCT-3'	IFN-5	5'-ATCGTTCAGCTGCTGGTACA-3'	IFN-6	5'-CCTCACAGCCAGGATGCTAT-3'
IFN-F	5'-GCTGTGAGGAAGTACTTTCAGAGGATCACCCTGTACCTGAAGGAGAAGAAGTACAGCCCTTGCGCTTGGGAAGTCGTGAGGG-3'	IFN-6	5'-CCTCACAGCCAGGATGCTAT-3'	IFN-7	5'-ATGATCTCAGCCCTCACGAC-3'
IFN-G	5'-CTGAGATCATGAGGAGCTTTAGCCTGAGCACCAACCTGCAAGAGAGCTTGAGGTCTAAGGAGTAA-3'	IFN-7	5'-ATGATCTCAGCCCTCACGAC-3'	IFN-8	5'-TGCTCTAGACTTTTTACTCCTTAGACCTCAAGCTCT-3'